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Gene Targeting in Kidney Development

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Cancer and development are conceptually related because tumor formation in many cases results from the aberrant expression of a developmental program. This is certainly true of Wilms' tumors, which display a range of phenotypes resembling various stages of kidney development. *WT1* has been identified as a tumor suppressor gene involved in a subset of Wilms' tumors. Gene targeting of the *WT1* gene demonstrated the requirement for this gene product during early urogenital development. Several other genes, including *Wnt-4, c-ret, ld* and *lim 1,* have been shown by gene targeting to also be involved in early kidney development. This review discusses gene targeting as an approach to the study of development and reviews the phenotypes of these and other genes involved in kidney organogenesis.

Key words: gene targeting, gene knockout, kidney development, Wilms' tumor

INTRODUCTION

Cancer may be understood as the aberrant expression of a developmental program. Tumors occur because of mutations in genes involved in the control of cell growth and differentation that render cells unable to complete a developmental program. Instead cells harboring these mutation(s) remain in an immature state and continue to divide uncontrollably. It is therefore important to study those genes that have been demonstrated to have roles in both neoplastic and developmental processes. Among this group of genes are some members of the growing family of tumor suppressor genes, which are defined by the observation that tumors arise as a consequence of a cell within an individual losing both copies of the gene and consequently undergoing neoplastic transformation. In addition to *WT1,* this family includes genes such as *p53* and *RB* which are both important regulators of cell growth.

Wilms' tumors are particularly interesting from a developmental perspective because unlike adult tumors of the kidney or other organs, which generally are derived from and resemble one particular cell type, Wilms' tumors often display a variety of cell types and arrangements that are characteristic of the normal kidney, but in a disorganized pattern. Some Wilms' tumors are predominantly blastemal in character, and others more epithelial. Sometimes tissues not normally found in the kidney, such as muscle, adipose tissue, cartilage, and bone are found, indicating that these tumors most likely originate from the primitive totipotent blastemal cells that are the progenitor of the renal epithelium and stroma, or perhaps from an even more primitive cell type. It is likely, therefore, that the molecular events that lead to Wilms' tumors have an important bearing on normal kidney development, and that an understanding of tumorigenesis in the developing kidney would also lead to a better general understanding of the interrelationship of developmental and oncogenic processes.

The last decade has seen an enormous growth in our ability to understand the molecular biology of both embryogenesis and tumorigenesis. We are continuing to improve our understanding of how the molecular events responsible for these processes are related. For many years the mouse has served as a model system for the study of development and tumor formation. However, the technology for both introducing new mutations into mice, as well as for cloning genes responsible for previously described mutations, has recently undergone remarkable advances. This article will review the known mutations that affect kidney development in mice, with an emphasis on those mutations that have been introduced by gene targeting.

GENE TARGETING IN EMBRYONIC STEM CELLS

Gene targeting technology allows the modification or mutation of a gene at its chromosomal locus [1,2]. Gene targeting or "knockouts" makes use of techniques developed over the past 15 years, which allow mutations to be specifically targeted to a particular gene in embryonic stem cells in culture, and then allows genetically manipulated cells to be introduced back into the mouse, so that the mutation can be studied in the whole organism. The

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advantages of this technology over studying mutated genes in transgenic mice that are traditionally derived by pronuclear injection are as follows. First, the mutation is targeted to a gene at its locus on the chromosome, abrogating questions about whether different integration sites affect the expression of different transgenes. Because the gene is mutated at its own locus, there is no change in the copy number of the gene, so that when mice are derived carrying the mutation, true dominant or recessive effects of the mutation are observed.

Embryonic stem (ES) cells are cell lines derived from the inner cell mass of the preimplantation mouse blastocyst; this is the population of cells from which the entire embryo proper is derived [1,3,4]. ES cells have the unique property of being able to be propagated in tissue culture, after which they can be introduced into the preimplantation blastocyst by microinjection to contribute to the group of cells comprising the inner cell mass. They can be genetically manipulated in culture by transfecting into them vectors prepared through recombinant DNA techniques [2,5,6]. In the most basic of several schemes, a "replacement" type vector is constructed by taking a cloned segment of genomic DNA of the gene to be studied, usually between 5 and 20 kb, and modifying this segment by removing an internal restriction fragment containing an important exon and inserting in its place a fragment which contains a gene (*Neo*) coding for resistance to an antibiotic normally toxic to ES cells, such as G418, an analog of neomycin [2,5,6]. When this vector is introduced into ES cells in culture by gene transfer techniques, usually electroporation, it will at some variable frequency undergo homologous recombination with the chromosomal locus of the gene of interest (see Fig. 1). When a homologous recombination event occurs, with a crossover occurring at both the upstream and downstream flanks, i.e., on either side of the antibiotic resistance gene, then the chromosomal gene becomes mutated by virute of having that important exon replaced by the resistance gene. The ES cells can be cloned and screened to find those that have undergone a true homologous event, rather than having become resistant through an illegitimate recombination of the vector randomly into a chromosome. The true homologous recombinants are selected for injection into mouse blastocysts.

Upon introduction into the blastocyst, ES cells will aggregate with the cells of the inner cell mass, such that when the injected blastocyst is reintroduced into the uterus of a pregnant mouse, the newborn mouse derived from that blastocyst will be chimeric, i.e., partially derived from the injected ES cells. When the ES cells contribute to the formation of germ cells of the chimera, either spermatocytes in a male chimera or ova in a female, any mutation originally placed in the injected ES cells will then be passed through the germline to the progeny of the original chimera. These progeny are then heterozygous for the mutation, and will demonstrate any dominant effects of the mutation. Furthermore, the heterozygotes can be intercrossed to determine if there is a recessive phenotype. If, for example, a particular gene is required for the formation of an organ, then mice homozygous for a mutation in the gene may develop with abnormalities in that organ, which can be studied further [5].

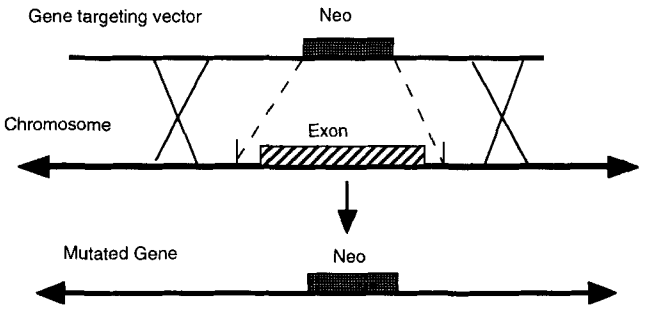

Fig. 1. Replacement vector for gene targeting. The gene targeting vector is homologous to the chromosomal DNA except for the sequence between the two restriction sites bordering the exon to be deleted. The dashed vertical lines indicate the breaks in homology. The solid vertical lines indicate the sites of homologous recombination of either side of the exon, resulting in the deletion of the exon and the introduction of the neomycin resistance gene into the chromosome.

KIDNEY DEVELOPMENT

Urogenital development in mammals proceeds in three stages, which are all characterized by the induction of a mesenchymal to epithelial transformation (reviewed in [8]). In the first two stages, the transient pronephros and mesonephros are induced along the urogenital ridge where nephrogenic mesenchyme is induced by the adjacent nephric duct to form nephric tubules. In the third stage, the definitive kidney forms as the result of subsequent reciprocal inductive interactions between the metanephric blastema and the ureteric bud, an outgrowth of the wolffian duct, during which the blastemal mesenchyme condenses around the bud and differentiates into renal epithelium. Nephric tubules, or nephrons, are the functional unit of the kidney and consist of the renal corpuscle, proximal and distal tubules which are derived from the metanephric blastema, and the collecting duct which is derived from the ureteric bud. In a series of organ culture experiments that defined the epithelial-mesenchymal interactions involved in formation of the metanephros, Grobstein [9,10] established the developing kidney as a classical system for the study of embryonic induction. In these experiments the explanted ureteric bud induces the mesenchymal to epithelial transformation while the mesenchyme is required for continued growth and branching of the bud. In addition to the ureteric bud, a limited number of tissues such as spinal cord have been shown in vitro to induce efficiently the differentiation of metanephric mesenchyme [9–11].

MUTATIONS DERIVED BY GENE TARGETING THAT AFFECT KIDNEY DEVELOPMENT

Several mutations have now been described whose phenotypes include defective kidney development. The mutated genes include *WT1, c-ret, Wnt-4, lim-1,* and *ld.* In addition, a transgenic mouse that misexpressed *Pax-2* has a kidney phenotype.

DEFECT OF MESENCHYMAL CELLS—*WT1* MUTANT MICE

WT-1 is a putative transcription factor containing four zinc fingers [12]. In experiments performed in tissue culture *WT-1* has been shown to repress the expression of a variety of genes sharing a common GC-rich motif in their promoter regions [13–18]. Studies in humans have shown that individuals constitutionally heterozygous for null mutations of the *WT1* gene experience cryptochidism (undescended testicles) and hypospadias (misplaced urethra) [19,20]. Included among these heterozygotes are individuals with the WAGR syndrome (*W*ilms' tumor, *a*niridia, *g*enitourinary malformation, mental *r*etardation), containing cytogenetically visible chromosomal deletions that affect the structural integrity of the *WT1* gene. In a second syndrome, Denys-Drash [21,22], hypertrophy of the podocyte cells within shrunken glomerular tufts and accumulation of matrix material in the mesangium produce a glomerulosclerosis that leads to a progressive renal failure at an early age [23]; this syndrome appears to be caused by dominant point mutations that affect the *WT1* zinc fingers [24–27]. Denys-Drash individuals also experience more severe genital malformations including ambiguous external genitalia and dysgenic or streak gonads. Both of these syndromes are associated with a predisposition to Wilms' tumors, and, consistent with Knudson's model for genetic predisposition to cancer [28], loss of heterozygosity occurs at the *WT1* locus in the tumor tissue [29]. Recently, mutations in *WT1* were identified in nephrogenic "rests", areas of blastemal hyperplasia that are believed to be the precursors of Wilms' tumors [30–32]. These observations define *WT1* as a tumor suppressor gene that plays an important role in urogenital development.

A mutation in the *WT1* gene, which deleted exon 1, coding for most of the proline-glutamine-rich amino terminal domain, was targeted in embryonic stem cells, and then introduced into the mouse germline [33]. Unlike humans heterozygous for constitutional mutation of one *WT1* gene, heterozygous mice did not develop kidney tumors. This is believed to be due to the 1,000-fold smaller target size of the mouse kidney compared with the human.

Homozygous mutant embryos died at embryonic days 13–15, probably of heart failure. The most dramatic consequence of the *WT1* mutation was the failure of kidney development. The Wolffian duct evolved normally along the length of the urogenital ridge in embryonic day 9-10 homozygous mutant embryos, however on embryonic day 11, the ureteric bud failed to grow out of the Wolffian duct towards the blastemal cells. The metanephric blastema was present in the *WT1* homozygous mutants; but the blastema of mutant embryos at day 11 revealed ongoing apoptosis. Although the nonapoptotic blastemal cells in the mutant appeared morphologically identical to blastemal cells in wild-type embryos prior to condensation around the ureteric bud, the mutant metanephric blastema completely degenerated and no mesenchymal cells were detectable by embryonic day 12. At later times, when all stages of tubular differentiation were distinguishable in the normal kidney, Wolffian ducts lying in a small ridge along the posterior abdomen represented the sole remnants of the urogenital system in mutant embryos [33]. (These results are summarized in the model presented in Fig. 2)

Analysis of the *WT1* Mutation in Embryonic Organ Culture

Metanephric mesenchyme obtained from mutant embryos was not induced by the spinal cord, while mesenchyme from wild-type embryos was induced in these experiments. The phenotype of the *WT1* mutation described above does not distinguish whether blastemal cell death occurs due to an autonomous cell defect or whether it is due to the absence of the normal inducer, namely the ureteric bud. In order to resolve this question, kidney rudiments from mutant and wild-type embryos were placed in culture. Duplicating the in vivo result, the mutant kidney was incapable of induction or growth when explanted alone, whereas the normal kidney differentiated and grew well in culture. Embryonic spinal cord, the strongest known in vitro inducer of tubular differentiation, was also used in an attempt to rescue the mutant metanephric mesenchyme, and was found to be unable to induce tubule formation in the mutant blastema [33]. Further studies have shown that the wild-type ureteric bud is also unable to induce mutant mesenchyme in organ culture. In the converse experiment, Wolffian ducts isolated from mutant embryos were shown to induce and send ureteric bud-like structures into the wild-type metanephric blastema (J. Kreidberg, unpublished results).

Wnt-4 DEFICIENT MICE

Wnt-4 is a member of the Wnt family of secreted glycoproteins, This family was identified by the observation that the prototypic member, *Wnt-1,* was activated as an oncogene in mammary tumors in mice. This family of genes was then found to have homology to the wingless gene which is a secreted or cell surface protein prominently involved in cell-cell signaling that determines cell

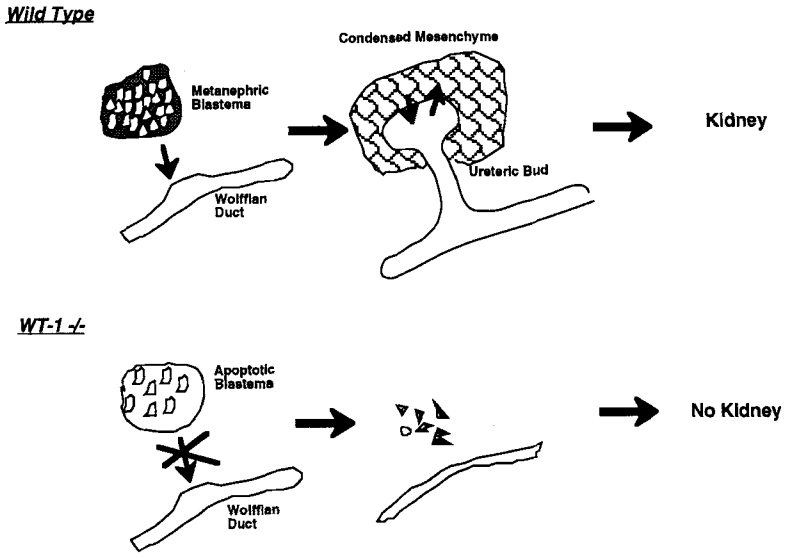

Fig. 2. Lack of kidney formation in WT-1−/− embryos. In the wild-type ureteric bud, outgrowth makes contact with the metanephric mesenchyme and induces the condensed mesenchyme to undergo a mesenchymal to epithelial transformation. In the mutant, the mesenchymal cells become apoptotic, and ureteric bud outgrowth does not occur. No kidney develops in the mutant.

fate during development in *Drosophila.* Wnt-4 is expressed in condensed mesenchyme and peritubular aggregates of the metanephric kidney, and continues to be expressed in a subset of epithelial cells of the comma and s-shaped tubules near the point of fusion with the collecting duct. No expression is seen in the mature nephron [34].

Stark et al. [34] have shown that kidney development is blocked at an early stage in *Wnt-4*-deficient mice. In the absence of *Wnt-4* the metanephric blastemal cells do aggregate around the ureteric bud, but fail to progress beyond that point. No peritubular aggregates or early tubules form. By embryonic day 15 growth of the kidney is retarded, and by birth only a very small nearly agenic kidney was apparent (summarized in Fig. 3).

In contrast to the *WT1* mutation, early outgrowth of the ureteric bud occurs and the ureter is able to undergo several branchings in the *Wnt-4* mutant embryo. *WT1* is indeed expressed in *Wnt-4*−/− kidneys, as are some other early markers of metanephric differentiation, including c-ret, N-Myc, and *Pax-2,* leading the authors to conclude that *Wnt-4* appears not to be required for the appearance or early condensation of the blastemal cells, but rather is involved in the subsequent induction of the mesenchymal to epithelial transformation [34]. It will therefore be of great interest to determine whether *WT1* or *Pax-2* is involved in the transcriptional regulation of *Wnt-4.*

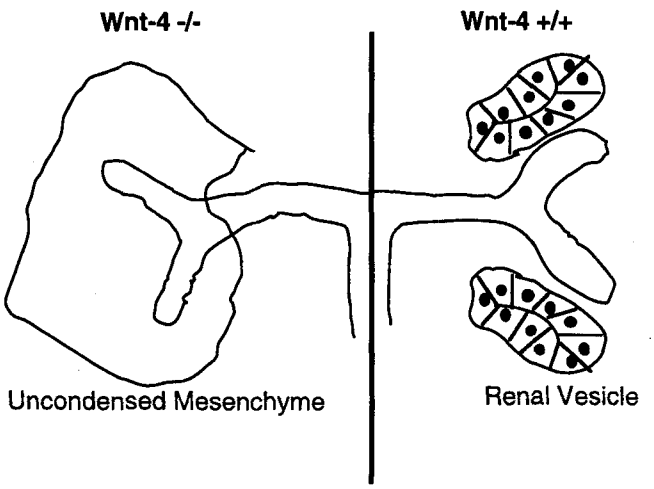

Fig. 3. A comparison of Wnt-4−/− and +/+ metanephric kidneys. Ureteric bud outgrowth occurs in both cases. In the mutant, the metanephric mesenchyme fails to condense into peritubular aggregates and undergoes induction. In the wild type, the peritubular aggregates are transformed into an epithelial vesicle which forms the tubule. The ureteric bud undergoes the first few branchings in the absence of Wnt-4, and then kidney development ceases.

DEFECTS IN URETERIC BUD OUTGROWTH: MUTATIONS IN c-ret AND ld

c-ret is a receptor tyrosine kinase expressed at the tip of the ureteric bud [35]. It has been identified as a proto-oncogene associated with multiple endocrine neoplasia 2A and medullary thyroid carcinoma. Schuchardt et al. [36] have developed c-ret mutant mice which are deficient in kidney development. There is some variabilty of this phenotype, mainly relating to the degree of ureteric bud outgrowth, which can range from both ureters reaching the kidneys to the complete absence of either ureter (Fig. 4). Consequently there is also variability in the degree of kidney formation, ranging from the formation of a

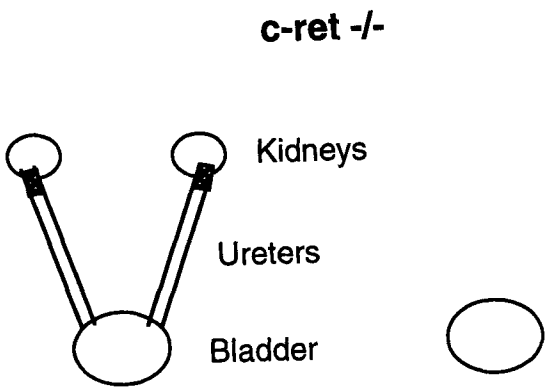

Fig. 4. Kidney development in c-ret −/− mice. The site of c-ret expression at the end of the ureteric bud is shown in black. The spectrum of phenotypes is shown, which ranges from complete bilateral renal agenesis, shown on the right, to both ureteric buds making contact with the metanephric mesenchyme and inducing small kidneys with a few tubules. Intermediates between these two extremes are common. A similar pattern is observed with both the ld and Sd mutations.

small kidney bilaterally, each with a few tubules and glomeruli, to bilateral renal agenesis. In no case did the kidneys in the mutants grow beyond a very small size compared with normal kidneys [36].

This variability suggests that ureteric bud outgrowth may require the function of at least one of a group of receptors during the initiation of metanephric development. In the absence of c-Ret, another receptor may be able to substitute to presumably receive a signal from the metanephric mesenchyme. A candidate alternate receptor is c-ros, another receptor tyrosine kinase expressed at the tip of the ureteric bud [37]. However, this substitution is clearly insufficient to provide full kidney development. Whether this is due to the substituting receptor transducing a much weaker signal, or not being expressed with sufficient duration, is a matter for further investigation. That in some cases no ureteric bud at all is observed suggests that there is a critical period at the beginning of metanephric development during which a sufficient signal must be received and transduced by c-ret, or ureteric bud outgrowth will not initiate.

ld, or limb deformity, is a mutation affecting both limb formation and urogenital development [38]. The gene was identified by the observation in a strain of transgenic mice that insertion of the transgene resulted in autosomal recessive syndactly and radioulnar fusion. This mutation was then found to be allelic with a known mouse limb formation mutant known as ld [38]. Further analysis of the mutant phenotype revealed that the homozygous mutants have incompletely penetrant renal agenesis [39]. The *ld* gene encodes a protein or group of proteins that have been named formins [40]. The function of the formins is unknown at this time.

Maas et al. [39] characterized the renal abnormalities in mice homozygous for five known alleles of *ld* that differ in their severity. As was observed for c-ret, there is variability in the phenotypes, ranging from bilateral renal agenesis to one normal and one hypoplastic kidney. Outgrowth of the ureteric bud is seen in many of the mutant embryos, however it either did not reach the metanephric blastemal cells, or reached those cells but then failed to begin branching or induce tubulogenesis. Interestingly, Maas et al. [39] showed that differentiation of the mutant blastema cells could be rescued by the embryonic spinal cord, a potent inducer of tubulogenesis in organ culture experiments. Therefore, ld expression is probably mainly required by the ureteric bud, even though ld mRNA was shown to be present in the mesenchymal cells as well.

MUTATION OF PDGF-B AND PDGF-β RECEPTOR AFFECTS KIDNEY DEVELOPMENT

A pair of papers showing in one case targeted mutation of the PDGF-B chain gene and in the other the PDGF-β receptor gene both demonstrated similar kidney defects [41,42]. Both mutations were lethal in the perinatal period, probably due to vascular defects. The kidneys were small, and no mesangial cells could be detected in glomeruli. A small number of podocytes were present in the glomeruli, but the glomerular tufts were essentially absent and the glomerular space was filled with one or a few distended capillary loops [41,42]. These papers indicate that signaling by PDGF is likely to be important in growth of mesangial cells, and indeed mesangial cells have been shown to produce PDGF and proliferate in response to this growth factor [43].

OTHER MUTATIONS AFFECTING KIDNEY DEVELOPMENT

Several groups have independently published a targeted mutation of the *N-myc* gene [44–47]. *N-myc* begins to be expressed in condensed blastemal cells during induction of tubulogenesis, and continues to be expressed in early tubules [48,49]. The *N-myc*-deficient embryos die at embryonic day 11.5, before the onset of metanephric development, however earlier stage mutant embryos show an inability of the mesonephric tubules to fully develop.

The Lim-1-deficient mouse has recently been reported with the striking phenotype of lacking formation of the head and anterior neural structures [50]. Lim-1 is a LIM-homeodomain protein expressed in the developing nervous and urogenital systems [51,52]. Most of these mutants died around embryonic day 10, prior to the onset of urogenital development. The few that survived to birth (without heads) were also reported to lack kidneys. The nature of the defect has not yet been investigated, since less than 1% of homozygous mutant embryos actually survived to birth [50].

Dressler et al.]52] reported observing abnormal kidneys in mice transgenic for the *Pax-2* gene. *Pax-2* is normally expressed in the early condensed metanephric

blastema, and then decreases in abundance as expression of *WT1* increases [53–55]. Unlike *WT1*, *Pax-2* is also expressed in the ureteric bud and wolffian duct [54]. In mice where expression of *Pax-2* was deregulated by placing it under the control of a heterologous promoter, multifocal microcystic dilation of tubules is seen, along with abnormal glomeruli in which the podocytes lacked foot processes [52]. It will be of great interest to examine kidney development in Pax-2 mutant mice when they become available.

Danforth's short tail (Sd) is a classically described dominant mouse mutant for which a candidate gene has not yet been identified. Like several of the mutations described above, considerable variability is observed in the degree of ureteric bud outgrowth and kidney development [56]. A recent study of Sd embryos noted that in some cases the ureteric bud was present but no metanephric rudiment was present [57]. Therefore, it is not yet clear whether the mutation is acting primarily in the ureteric bud or metanephric blastema, or both.

MOUSE MUTANTS WITH POLYCYSTIC KIDNEY DISEASE (PKD)

Several spontaneous recessive mouse mutation strains as well as one strain derived by insertional mutagenesis in a transgenic mouse and one gene-targeted mouse strain have been reported with PKD. They are included in this review because in certain of these strains the condition is present at birth and may be considered as a developmental defect.

Mice homozygous for target disruption of the *Bcl-2* gene suffer from PKD [58,59]. *Bcl-2* is one of the major mediators of apoptosis. While apoptosis occurs at a low level during kidney development, it is not known why PKD develops in these mice.

A transgenic mouse obtained as part of a large-scale screen for mutants created by insertional mutagenesis was observed to develop cystic lesions which were present in the proximal tubules at birth and then progressively shifted to involve the collecting ducts during the first few weeks of life. A novel candidate gene that contained a motif found in genes involved in control of the cell cycle [60] was cloned from the transgene insertion site.

The spontaneously occurring mutations that give rise to PKD include the pcy, cpk, bpk, and jck strains of mice [61–64]. None of the genes responsible for these mutations has yet been clones, although the chromosomal locations have been delimited to varying degrees. These mutant lines have been used to study the different hypotheses offered to explain the development of PKD. These are generally placed in three groups [65]. 1) Cysts are the result of loss of polarity of the tubule cells, resulting in the misplacement of ion pumps on the apical surfaces of the cells. This leads to oversecretion of water and increased pressure within the lumen of the tubule. 2) Cysts are the result of increased cell division of tubule cells, resulting in large tubules. 3) Cysts are the result of defects in the extracellular matrix surrounding the tubules. There is experimental evidence for all of these possibilities, and it is possible that more than one etiology exists for the development of PKD.

PARADOXES IN EXPERIMENTAL KIDNEY DEVELOPMENT

Gene targeting experiments have led to paradoxical situations in the experimental study of kidney development. In several instances, particular growth factors had been shown in organ culture experiments to be required for proper kidney development [66,67]. Subsequently, kidney development was found to be normal in mice in which the genes coding for those growth factors had been deleted [68–72]. This includes transforming growth factor α, and the insulin growth factors 1 and 2. In these cases it is likely that the organ culture presented a more stringent setting for metanephric growth, whereas in the developing embryo there may be functional redundancy among groups of growth factors.

NOTE ADDED IN PROOF

Since the preparation of this manuscript, which covered reports prior to April 1, 1995, several additional gene targeting experiments have led to interesting kidney phenotypes. These genes include BMP-7 (OP-1) [73,74], Pax-2 [75], and BF-2 [76].

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COMMENTARY

Kreidberg starts off by providing an excellent description of gene targeting or "knockouts" in the section "Gene Targeting in Embryonic Cells." Once having explained the technique he uses, Kreidberg demonstrates how gene targeting has provided evidence for the involvement of several genes, including *WT1*, in early kidney development. For example, in mice in which embryonic stem cell with a homozygous deletion of *WT1* exon 1 are introduced, the renal mesenchymal cells become apoptotic, and ureteric bud outgrowth does not occur. As a consequence, the kidney fails to develop (Fig. 2). In the *Wnt-4* (another gene involved in renal development), deficient mice however, early outgrowth of the ureteric bud does occur, but the metanephric mesenchyme subsequently fails to condense in peritubular aggregates and undergoes induction (Fig. 3). Kreidberg's experiments allow a unique approach to the study of renal development and ultimately renal cancer, since the latter can be viewed as the aberrant expression of the first.